Gene Expression in the Early Development stages in Populations of European Grayling (*Thymallus thymallus*)

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Abstract

European grayling from a common-gene pool and with a known ancestry were released into headwaters of a river system in Lesja, Oppland, Norway, about a century ago. These grayling dispersed downstream, and as a result there are now several different populations in this river system, all influenced by different environmental factors. Earlier research has found these populations to differ in phenotype, and evidence for genetic structuring of the populations has been found. In this thesis I conduct a common-garden experiment with two temperatures to investigate early life-history traits in different populations of grayling, focusing on development and gene expression (RNA analysis with real-time PCR). For five populations the timing of hatching and eye pigment formation was observed, and for three of these populations I estimated relative mRNA-levels for three target genes; Tropomy, and heat-shock genes HSC70 and HSP70. I found no clear pattern of differences in timing of development rates, and there were no differences in expression of the genes between the populations. I suggest that these results are a consequence of pleiotropy, but find it unlikely for Tropomy and HSC70, due to plasticity shown for these genes. I do find significant differences in expression of Tropomy and HSC70 between temperatures. Individuals incubated at low temperature (6°C) had a higher expression of the Tropomy gene and HSC70 gene, than individuals incubated at high temperature (10°C). This plastic response indicates that the two genes are important for cold tolerance in grayling.

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Introduction

All species occurs in some kind of population structure that are often separated in geography and are in varying degrees, reproductively isolated from each other (Taylor 1991). When the environment of a population's habitat changes, or if a population colonize new habitats, these new conditions may cause strong selection pressure, and the differences in environmental factor's selection pressure between habitats may control phenotypic expression (Haugen 2000c; Haugen 2000a). Differences in local environments can result in populations being dissimilar in morphology, behavior, physiology and life-history strategies (Taylor 1991). Local adaptation can be defined as a process that increase the frequency of trait within a population, which enhances the survival or reproductive success of a individuals expressing these traits (Taylor 1991). The study of local adaptation is showing how selection is responsible for the development of a trait within a population and describing which selective pressure has favored it (Futuyma 1986). Fish are ectotherms, and thus water temperature is a vital environmental factor that will have a large effect on developmental processes, e.g. determine the rates of muscle formation (Taylor 1991; Johnston 2006). Miller and Brannon (1982) proclaimed that variation in local water temperature is a major selective factor that maintains variation between fish population in developmental biology. Both old and more recent studies support this statement where embryonic and larval survival and development rates from fertilization to emergence appeared to be an adaptive response to local water temperatures in species such as chinook salmon (*Oncorhynchus tshawytscha*), pink salmon (*Oncorhynchus gorbuscha*), and grayling (*Thymallus thymallus*). (Beacham 1988; Beacham and Murray 1988, 1989; Haugen 2000a; Haugen 2000b). Differences in development can be a plastic response to the water temperature of the different habitats. A plastic response is when a gene shows several different phenotype in response to different environmental conditions (West-Eberhard 1989). For plasticity to be adaptive, phenotype and environment must interact to increase individual fitness (Nunney & Cheung 1997).

European grayling (*Thymallus thymallus*) in a river system in Lesja, Oppland, Norway, all come from a common ancestor. These grayling that all stem from a recent common gene-pool were released into the headwaters of this system in 1910, and began to spread downstream and started to occupy different habitats (Haugen 2000a). The habitats had different environmental impacts on grayling, especially were they different in temperature, and a change in environmental factors might force a selective response in the different grayling

populations (Taylor 1991). Introduced populations with a known origin provide a chance to examine divergence in phenotypic traits (Hendry et al. 1998, Kinnison et al. 1998). In 2000 Thrond Haugen conducted a field experiment and saw that in the field the different populations of grayling varied in important fitness traits such as growth rate and survival during early life history stages. The field experiment documented significant differences between population in larval survival rates, differences in early growth rate, and this study provided evidence of rapid phenotypic divergence in the grayling populations that were exposed to different habitat environments and thus different selection pressure (Haugen 2000a). The populations also varied strongly in mortality rates and growth pattern (Haugen 2000b), and estimated evolutionary and divergence rates were found to be high compared to other similar studies (Haugen & Vøllestad 2001).

In later years additional experiments and research have been preformed on grayling in the river system in Lesja. It has previously been demonstrated that grayling in warmer, smaller streams have larger eggs compared with females of same body size spawning in colder, larger streams (Gregersen & Haugen 2008). Kavanagh et al. (2010) confirmed different embryo and larval development patterns between offspring from parents spawning in cold versus warm tributaries. Offspring from cold streams grew faster and had a higher yolk-to-body mass conversion efficiency then those from warmer streams (Kavanagh et al. 2010). Many good evidence of rapid adaptive phenotypic divergence induced by temperature has been provided from studies of the grayling in Lesja, which also indicates that the differences most likely also have a genetic basis (Haugen 2000b; Haugen & Vøllestad 2001; Thomassen et al. 2011). A weak, but significant isolation-by-distance population structure was detected in studies of populations from Lesjaskogsvatnet (Barson et al. 2009; Junge et al. 2011). Kavenagh et al. (2010) saw that the divergence of the populations was followed by adaptation; the populations inhabiting cold environments displayed higher growth rates and yolk conversion efficiency than populations of warmer habitats. They found that genetic based phenotypic divergence can happen even under conditions of low genetic variation and ongoing gene flow (Kavanagh et al. 2010).

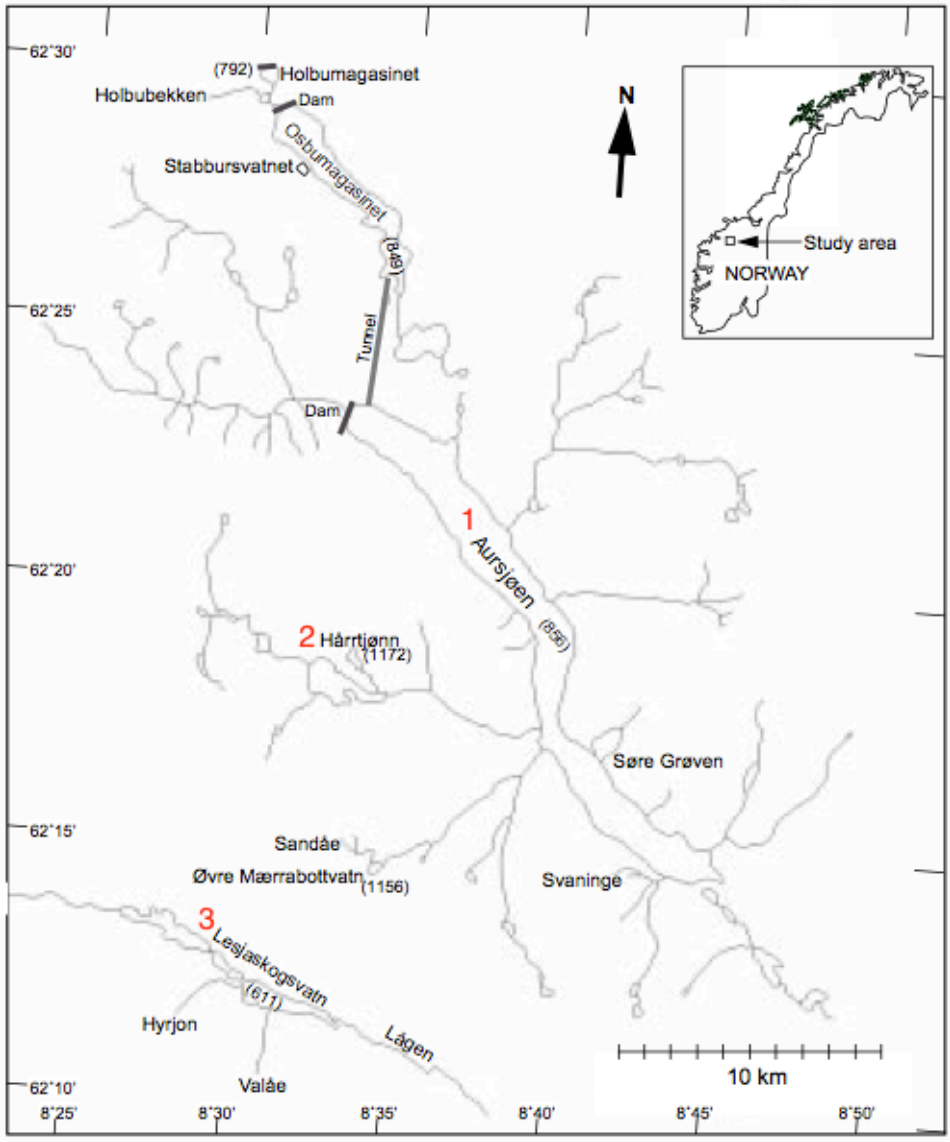
Evidence for both phenotypic and genetic adaptations between populations in Lesja has been established. The genetic differences found was at neutral loci (F_{ST}), and little is known about which genes are involved in forming these differences, and how gene expression varies between the populations.

In this thesis I will focus on variation in early development and level of gene expression between populations of grayling. Gametes have been sampled from five different populations in the field, and a crossing experiment was conducted (within each population), followed by a common-garden experiment were the fertilized eggs incubated at high and low temperatures. A dataset of five populations were formed from this to detect any differences in timing of eye pigment formation and hatching. A reduced dataset with three populations was analyzed (mRNA analysis with real-time PCR) in order to try to detect variations in early development rates and differences in gene expression between populations, at a fixed development stage. I examine three target genes to look for differences in plastic response and gene expression induced by temperature differences in incubation habitat. The three genes chosen are the two heat-shock genes HSP70 and HSC70, and a gene that codes for fast myotomal muscle tropomyosin mRNA, Tropomy. These genes has earlier been proven to be important for early development, and the expression of these genes has been found to be influenced by temperature (Krone et al. 1997; Montarras et al. 1982; Bandman et al. 1982). Heat-shock proteins have been found to be highly affected by differences in water temperature; even in seasonal variation of water temperature has researchers found significant differences in expression of heat-shock genes (Feder & Hofmann 1999). According to these findings, differences in gene expression should be possible to identify.

Materials and Methods

The Study Organism and Area

The European grayling is a member of the family Salmonidae (Hendry & Stearns 2004), and is easily recognized by its tall dorsal fin, which is larger in males than females and often shines in green, red and blue. Grayling are distributed throughout Europe (Northcote 1995) in cold, oxygen-rich rivers with rapid currents as well as in lakes (Muus 1968).



Figur 1: Study area. Two of the populations sampled from are tributaties of Lesjaskogsvatnet (3); Steinbekken and Valåa. The third population sampled from was Kvita, a tributary of Aursjøen (1). Samples from Hårrtjønn was also included (2), and the last population sampled from was Gudbrandsdalslågen in Otta. From the map, Gudbrandsdalslågen can be seen as the eastern outlet river from Lesjaskogsvatnet. Sampling was done downstream in Otta. Map is from Haugen (2000a)

In Norway, grayling are naturally distributed in the eastern parts of northern Norway (Finnmark) and in larger river systems in the southeastern part of the country (Pethon & Nystrøm 1985). Northcote published a review of the biology of grayling in 1995, where he describes the European grayling to be a spring-spawner that spawns during late March to June. The spawning behavior of European grayling is slightly different from that of the other salmonids. The male set up and sustain spawning territories, rather than fighting for access to the females, which is common in other salmonids (Northcote 1995). The females only enter the spawning area for a short period of time to spawn, and they don't construct "nests" for the eggs like other salmonids (Northcote 1995). The grayling eggs usually lay at a depth of 2-3 cm in the gravel. Development is temperature dependent, and hatching usually occur after 180 to 200 degree-days (Haugen & Vøllestad 2000).The grayling population in Lesjaskogsvatn, Oppland central Norway, was established in the late 1880s (Haugen 2000c). It is assumed that the fish originated from the river Gudbrandsdalslågen and that the fish invaded Lesjasjogsvatnet during a period with human activity in the out flowing part of the river. In 1910, fish from this population were released into Hårrtjønn and Øvre Mærrabottvatn and fish from these populations later dispersed into Aursjøen in the 1920's and into Osbu- and Holbumagasinet in the 1950s (fig. 1) (Haugen 2000c).

In this study I collected grayling gametes from 5 different populations (fig. 1). To represent the founder population, fish were collected from Gudbrandsdalslågen at Otta. Gametes were also sampled from Steinbekken and Valåa, two tributaries to Lesjaskogsvatnet (fig. 1). Steinbekken is categorized as being warm during spring, whereas Valåa is cold. Kvita is a tributary to the lake Aursjøen – a large lake situated at 856 m above sea level (fig. 1). The last population that was sampled from was Hårrtjønn, a warm, small high-mountain lake situated 1172 meters above sea level (fig. 1) (Haugen 2000a)

Field Sampling and Experimental Design

In the field, adult grayling were caught during spawning or during spawning migration by fishing with hook and line (in Gudbrandsdalslågen), and using traps and fyke nets set up in the streams (Hårrtjønn, Kvita, Valåa and Steinbekken; fig 1). When caught, the fish were transferred to holding pens. Before retaining the eggs and sperm, the grayling were anesthetized using benzocaine. Once the fish was sedated, eggs and sperm were collected in individual plastic bags. Afterwards, the fish was put into a tank with fresh water for recovery, and once awakened they were released into the stream. The plastic bags containing gametes

were stored on ice in containers for transportation by car to The Department of Bioscience at the University of Oslo (see appendix 1).

When in the lab, the crossing experiment was conducted. A standard volume of egg from every female from each population was pooled for randomization. The eggs were then split into a number of batches equal to the number of males from that particular population. Each batch was fertilized with sperm from one male to avoid sperm competition. After 3-5 minutes, the mixture of sperm and eggs were rinsed with water. These fertilized eggs were then carefully put into individual wells on standard 24-well culture plates with water added. A total of 216 eggs on 9 plates per population were then distributed into three different culture rooms for incubation. Target temperatures was 6 °C, 8 °C and 10 °C. These target temperatures were chosen, partly to represent cold, medium and warm temperatures experienced by developing grayling larvae in nature, and partly because of limitations of the capability to manipulate temperatures due to technical constraints. The same temperatures have been used in a number of other similar experiments on grayling from the same area (Thomassen et al. 2011; Kavanagh et al. 2010). Temperatures were continuously monitored using HOBO data loggers. The climate rooms where not as stable as we had expected, therefore the actual temperature was different from what we predicted. The 8°C had an average temperature of 7°C, the temperature room of 6°C had an average temperature of 6,7°C, and the 10°C room had an average temperature of 10,23°C. Because of this we only went further with two temperatures: low (between 6°C and 7°C) and high (10°C).

Sampling of the developing embryos was done at 4 different stages: I) eye pigment formation, II) a fixed number of degree-day close to eye pigmentation (approximately 140), III) a fixed number of degree-day close to hatching (approximately 164) , and IV) the last sampling was done when the egg hatched (see Appendix 2). The fixed number of degree-days for sampling was estimated based on the results from earlier experiments (Thomassen 2009; Thomassen 2011). This sampling schedule was made to get samples for several potential sub-projects (focusing on both gene and protein expression). Only parts of the material sampled are used here. The exact number of degree-days at sampling was calculated by multiplying the number of days with the average temperature in the current time period. The sampled eggs and embryos were put individually into tubes and quickly frozen on dry ice before being transferred to an -80°C freezer for storage. Some water was unfortunately always included with the embryos.

RNA Extraction and cDNA Synthesis

Only a subset of the embryos from time point III and three selected populations (Otta, Kvita and Valåa) were selected for analysis of gene expression. This was due to a problem encountered with the RNA extraction. The eggs were often frozen together with a small volume of water, and I had a hard time getting them quickly enough out of the ice and into the Tri Reagent for RNA-extraction. When analyzed, a large amount of samples were too degraded for further cDNA synthesis and qPCR. Three temperatures were originally selected for this experiment, but only two were used for gene expression (high and low) due to similarity between the two lowest temperatures (see above).

The eggs and larvae frozen were thawed on ice and chopped out of the remaining ice in order to place them as quickly as possible into the Tri Reagent (Ambion® RNA, Life Technologies). The RNA extraction kit TRI Reagent® Solution from Ambion was used in all the extractions according to the manufactures protocol (Ambion® RNA, Life Technologies). The RNA extraction consists of a first and second extraction step. The result of the first extraction is a liquid separated into three layers. The upper transparent aquatic layer contains RNA and remaining two layers of liquids are proteins and DNA. RNA is pipette into fresh tubes with isopropanol so that the RNA forms as a pellet in the bottom. Supernatant is discarded and the RNA is stored with ethanol in freezer. The RNA is then DNase treated befor the second extraction, which is identical to the first extraction, only with smaller amount of reagents.

They where then diluted and the RNA concentration was measured using The NanoDrop; 1000 Spectrophotometer from Thermo Scientific (Thermo Fisher Scientific, Inc). Furthermore, the sample's RNA quality and integrity were measured using the Agilent Bioanalyzer (©Aligent Technologies). The BioAnalyser reports a integrity number (RIN) which is calculated by an algorithm that takes characteristics of numerous regions of the recorded electropherogram into account (Schroeder et al. 2006). A high RIN value signifies a sample with very little depredated RNA, and a low RIN value signifies RNA that is very degraded. All samples used in qPCR had a high RIN value that varied between 8 and 9,90, and a electropherogram with a clear peak for both the 18s and 28s ribosomal units. For the cDNA synthesis, the iScript™ cDNA synthesis Kit from Bio-Rad was used (©Bio-Rad

Laboratories, Inc). This kit comes with iScript reverse transcriptase, 5x iScript reaction mix and nuclease free water. Each one of these reagents where used to make a cDNA synthesis master mix that were added to the RNA used. cDNA synthesis was carried out according to the manufactures' protocol (©Bio-Rad Laboratories, Inc).

Primers were ordered from Applied Biosystems for Tropomy, and HSC70 (see table 1), and were diluted with water according to the manufactures directions. The primers for the last target gene HSP70, and the two reference genes 18s and EF1, I received from the laboratory of the genetics department at the University in Turku, which they had left over from previous experiments. When choosing which genes to study, there were three factors that were crucial; how they responded to temperature, the gene's function in early development, and availability of primers. To test the functionality of the cDNA from the grayling embryos with the selected primers, I ran PCR and agarose gel electrophoresis. The purpose of this was to test that the cDNA actually was synthesized, and that the primers we had chosen was able to work with the cDNA. For the PCR, a standard master mix with buffer, $MgCl_2$, dNTP, enzyme and water was made. The master mix was then mixed with primer pairs and cDNA before running the PCR on the C1000 Thermal Cycler from Bio-Rad Laboratories (©Bio-Rad Laboratories, Inc). An agarose 2% gel was made for the electrophoresis, which was performed on the Molecular Imager® Gel Doc™ System from Bio-Rad Laboratories, was used to capture and adjust the image of the gel (©Bio-Rad Laboratories, Inc).

Table 1: Primer name and sequences from Applied Biosystem

Primer name	**Sekvenes 5' - 3'**
HSC70 F	CGGCATCATGAACGTGTCC
HSC70 R	CTTGTCACGCTGCACATCAT
Tropomy F	GACCAACAACCTGAAGTCACTG
Tropomy R	TCAATGGTCTTCTCAAGTTTGGC

qPRC: Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction, or qPCR is a detection and quantification tool used in the laboratory. qPCR uses fluorescent reporter molecules to observe the production of amplification products during each cycle of the PCR (Bustin & Mueller 2005). The

fluorescent reagent I used were Fast SYBR® Green from Applied Biosystems (Applied Biosystems®, Life Technologies, Inc). The unbound SYBR Green dye shows slight fluorescence, but during PCR the dye binds to double stranded DNA, and when monitored in real-time it results in an increase in the fluorescence signal as the PCR proceeds. Preparations for qPCR included making a SYBR Green and primer master mix were the mixture consisted of 5ul SYBR Green master mix, 1ul F primer and 1ul R primer. This was thereafter loaded in to the wells in the qPCR plate. For a big plate using 5 genes this master mix was made for 74 wells (number of wells per gene). 1ul cDNA with 2ul H_20 was also added to the wells before running the qPCR. 18 cDNA samples, with 15 wells for each cDNA sample, and two plates were run. Included on the qPCR plate a standard curve test with dilution from 1.0 to 0.03 cDNA was added. The standard curve method uses a set of relative standards from which unidentified samples are quantified. The dilution is known, and the melting curve should show a low C_T value for the 1.0 dilution, and a much higher for the 0.03 dilutions. The CT is the threshold cycle, the cycle number at which the fluorescence generated within a reaction crosses the threshold line and starts an exponential increase in DNA generated (Applied Biosystems, Life Technologies, Inc. 2006).

The qPCR was carried out at Turku Center for Biotechnology using the 7900HT Fast Real-Time PCR machine from Applied Biosystems, running the ABI 384-well plates (Applied Biosystems®, Life Technologies, Inc). All samples and master mix were prepared and loaded in the afternoon before leaving it in the qPCR in a cold room over night. Two 384-well qPCR plates were run. On plate one, three individuals from each population and temperature were measured for the three target genes and two reference genes, while on the second plate some individuals were run again, and new ones were added (see Appendix 4).

Data Analysis

Real-time PCR were conducted and the data were analyzed using the QuantStudio™ 12K Flex Software (Applied Biosystems®, Life Technologies, Inc). The relative quantification method was used where an active reference is used to determine changes in the amount of a given sample relative to another internal control sample (Applied Biosystems, Life Technologies, Inc. 2006). The mRNA expression levels of genes were recorded as Ct values that corresponded to the number of cycles at which the fluorescence signal can be detected above a threshold value. Baseline and threshold for Ct calculation were set automatically using

the QuantStudio™ 12K Flex Software v1.2.1 (Applied Biosystems®, Life Technologies, Inc). Conversion of Ct values to relative quantities was done using standard curves. Standard curves were constructed from dilution series of pooled cDNA (including five dilutions from 1.0 to 0.03) (see appendix 4), and the PCR efficiency was calculated using the equation E% = $(10^{1/slope} - 1) \times 100$. Data of each target gene were then normalized with the geometric mean of the two reference genes. Slope/amplification efficiency values were calculated by using the slope of the regression line in the standard curve. A slope close to -3.3 indicates optimal PCR amplification efficiency, and the R^2 values should be >0.99, which represents a perfect fit between the regression line and the data points (Applied Biosystems, Life Technologies, Inc., 2006).

In Rstudio (© 2014 RStudio, Inc.) a generalized linear mixed model (GLMM) analyses were done using the R package "Linear mixed-effects models using S4 classes" (lme4), together with the R language package (© 2014 RStudio, Inc). GLMMs merge the assets of two statistical frameworks, linear mixed models (which include random effects), and generalized linear models, which handle non-normal data by using link functions and exponential family distributions (Bolker et al. 2009). Model selection was done using the Akaike Information Criterion (AIC), which finds the most parsimonious model as a balance between variations explained by the model (decreasing values) and the number of parameters included (increasing values) (Burn-ham & Anderson 2002). Several GLMMs was conducted and the most parsimonious model of mRNA expression between populations, the one with the lowest AIC-value, was when temperature and populations were not interacting. The model structure used for the GLMM was as follows: gene ~ Population + Temperature + (Plate|Individual), an equation with two explanatory variables: temperature (a continuous variable) and population (factor variable with three levels), and two random effects: Plate and Individual. When working with a factor variable, the level that starts with the letter that comes first in the alphabet, will be the one that the other levels are compared to, it is the reference. So in this case the GLMM is testing whether Otta and Valåa have a different gene expression from Kvita, the reference. This was the model structure that best fit the date based on AIC. A linear model analyses was also done for each gene using the model structure: Gene ~ Temperature + Population. The linear model was only conducted using the fist plate values. This was due to the evenly distributed number of replicates on this plate versus the second plate.

Genes of Interest

During qPCR we look for differences in expression of target genes, genes of interest. These are genes that hopefully show differences in expression when examining different populations that are exposed to similar environmental factors. Two of the target genes I investigated were heat shock genes. These are genes that codes for proteins that are present in all organisms that have been genetically examined, and in the unstressed cells these proteins have functions that are important for protein metabolism (Krone et al. 1997; Basu et al. 2002). When cells are exposed to stressful environmental factors like elevated temperature, this results in activation of the heat shock genes (Krone et al. 1997). There are three major families of heat shock proteins: Low molecular weight Heat-shock proteins, Heat-sock proteins 90 and Heat-shock proteins 70. It is in the latter family of the three that you find both of the heat-shock genes studied in this thesis; HSP70 and HSC70 (Basu et al. 2002). Heat shock genes shows a variety of patterns of spatial and temporal regulation during early embryonic development in a broad collection of organisms (Krone et al. 1997). Since Heat-shock are up-regulated when exposed to elevated temperatures (Tonsor et al. 2008), Because of this, these genes are good target gene for detecting genetic dissimilarities between populations that have been subjected to different habitat temperature when incubating. Heat shock genes codes for heat shock proteins, which again synthesize heat-shock cognates. These are protein chaperones that can disaggregate proteins from large aggregates or assemblies, prevent aggregation of denatured proteins, aid the renaturation or folding of proteins to reach their proper conformation, direct proteins to decomposing pathways, and bind protein to restrain their function (Gong & Golic 2005).

Heat-shock proteins are very studies, and some families have distinct activities, they also demonstrate overlapping roles, they cooperate in their activities and may share proteins that act as cofactors, known ass co-chaperones (Gong & Golic 2005; Smith 1993; Chen et al. 1996; Sanchez et al. 1993). HSP70 proteins function as chaperones, but they are also important in other activities. It function also as a protease when it is aided by co-chaperones; it can control substrate proteins to refold or to be degraded. The HSC70 CHIP (carboxyl-terminus of HSC70 protein) is a ubiquitin ligase, a post-translation modification, that directs substrate proteins to the proteasome, where the proteins are degraded (Gong & Golic 2005; Ballinger et al. 1999). Other activities of the HSP70 proteins are regulation of apoptosis (programmed cell death), and obtaining innate (non-specific) and adaptive (specific)

immunity (Gabai et al. 1997). Interest in the HSP70 family of chaperones is growing because of the large variety of cellular processes in which they are involved, as well as their possible participation in aging, cancer, and several neurodegenerative genetic disorders (Gong & Golic 2005). HSP70 are also involved in non-heat-shock processes. Recently it has been discovered that HSP70 can modulate the effect of expressing polyglutamine disease genes (Gong & Golic 2005; Warrick et al. 1999).

The third target gene is a gene coding for fast myotomal muscle tropomyosin mRNA, Tropomy. Tropomyosin together with a complex of troponin plays a vital role in muscle contraction (Montarras et al. 1982). Tropomyosin is a striated muscle regulatory protein, and forms a family of highly conserved actin binding proteins (Huang & Ochiai 2005). Tropomyosin is a two subunits molecule that forms a coiled-coil structure consisting of two parallel alpha-helical polypeptides (Crick 1953; Huang & Ochiai 2005). Earlier, changes in the tropomyosin subunits patterns during chick (*Gallus gallus domesticus*) embryonic development has been detected (Montarras et al. 1982; Bandman et al. 1982). The thermal stability of fish fast skeletal muscle tropomyosin has also been studied, and it was found that tropomyosin shows species-specific thermal stability (Huang & Ochiai 2005). Since this gene is affected by distortions in the temperature, it is a good starting point when it comes to examining differences in gene expression due to different temperature.

When doing qPCR analyses one need to have reference genes for comparison. Reference genes are used to make sure that the variation observed is caused by factors of interest, e.g. temperature, and therefore an ideal reference gene should be stably expressed across samples from different development stages (Kuchipudi et al. 2012). Recommendations on the use of two reference genes are given from Radonić et al. (2004), this is to obtain the most reliable results in gene transcription analysis (Radonić et al. 2004). Two reference genes were thus chosen: EF-1α and 18S-rRNA. EF-1α is a gene that endorses the GTP-dependent binding of aminocyl-tRNA to the ribosome (Uetsuki et al. 1989). It is a gene that has been used as a reference in other gene expression studies because of its stability in expression (McCairns & Bernatchez 2009). The other reference gene, 18S-rRNA, a gene that has shown to be a very a stable reference gene when using qPCR (Kuchipudi et al. 2012). The 18S gene is part of the ribosomal functional core, the ribosomal RNA, and is exposed to similar selective pressure in every living organism (Meyer et al. 2010).

Results

Timing of Eye Development and Hatching

The monitoring and sampling of the eggs was carried out over 39 days, from 5th of June to 14th of July. Figure 2 shows a bar chart of how many degree-days each population used to reach eye pigment formation, and how many degree-days to hatching. Steinbekken population used longer time developing eye pigment when incubating in low temperature, while its the exact opposite for the other four populations, which used more degree-days developing eye pigment when incubating in high temperature.

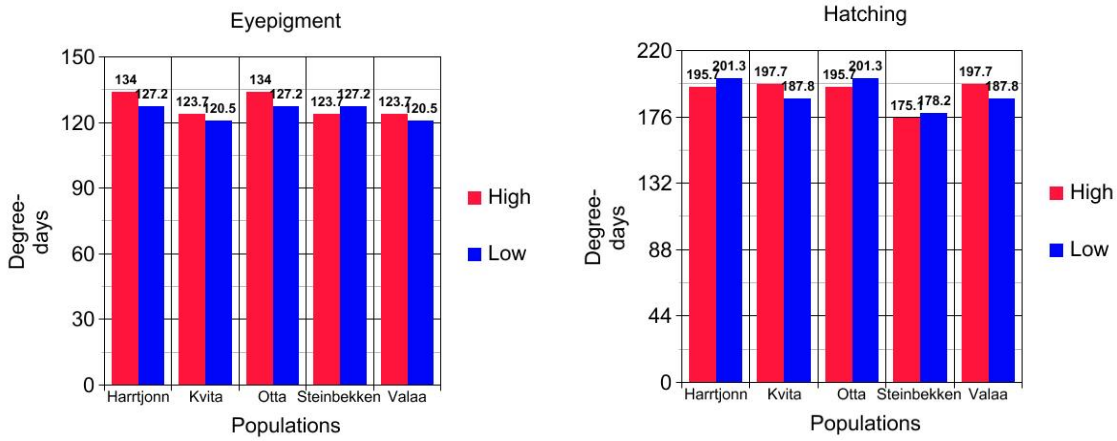


Figure 2: Number of degree-days used to reach the eye pigment formation stage and to hatching for grayling embryos from five populations incubated at high and low temperatures (see appendix 3 for details). The time is measured in degree-days (x-axis), and the red bars presents degree-days used when incubated in high temperature, while the blue bars presents degree-days used when incubated in low temperature. The populations can be found on the y-axis.

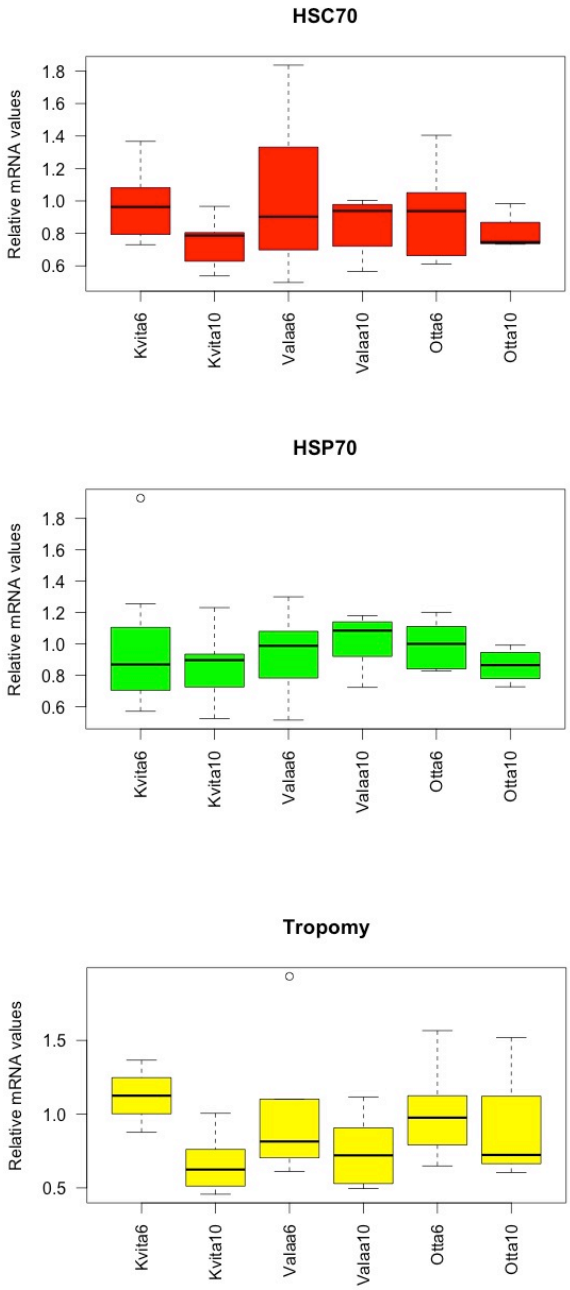
When hatching, Steinbekken, Otta and Hårrtjønn all used more degree-days when incubating in low temperature. Kvita and Valåa used more time to hatch when incubating in high temperature. Steinbekken was the population that clearly used fewest degree-days in both temperatures, to hatch. Note that this figure is in degree-days, and that the variation in timing of hatching and eye pigment formation has larger variation between population and between temperature treatments when measured in days.

Gene Expression

The total number of samples was not evenly distributed across populations and temperature, and thus the mean mRNA expression can vary more than it would if the sample numbers

where even across temperatures and populations. For the measurement of gene expression to be as accurate as possible, there must be little variation between reference gene expressions across populations and the two qPCR plates. To get a good measurement of the differences in gene expression between the populations, it is important that the two plates analysed varies in random differences as little as possible. GLMMs were conducted with plate as a random effect to account for random differences between the plates.

There was no clear pattern in the expression of Tropomy gene between populations. A generalized linear mixed model (GLMM) was conducted and there were no significant differences between the populations in expression of the Tropomy gene (table 2). There was also no effect of temperature treatment. The power of the GLMM is probably low due to the low sample size. A linear model (LM) was also done, using only the results from plate 1. The result from this analysis shows a significant difference in gene expression between temperatures. Individuals incubated at low (6°C) temperature had a higher expression of the Tropomy gene, than individuals incubated at high (10°C) temperature. This trend is most obviously for Kvita individuals.



Figur 3: Boxplot of relative mRNA expression of Tropomy (bottom), HSP70 and HSC70 (top), with two temperatues low and high and three populations, Kvita, Valåa and Otta.

Hints of the same pattern can be seen for the heat shock gene HSC70 (fig. 3). No clear pattern of expression of the gene between no significant differences between the populations was found in expression of the HSC70 gene (table 2). There was also no effect of temperature treatment. A linear model (LM) was also done, using only the results from plate 1. The result

from this analysis shows a significant difference in gene expression between temperatures. The HSC70 gene was also expressed at a higher level in individuals incubated at low temperature (6°C).

In the second heat shock gene HSP70, there is no clear pattern in the expression of the gene between population (fig. 3), and one can nor see a clear pattern of differences in gene expression between temperatures. Generalized linear mixed model and linear model was carried out for this gene as well, showing no significant differences between populations or between temperatures (see table 2).

Table 2: Summary statistics (parameter estimates ± 95% confidence interval) from Generalized linear mixed models (GLMM) and linear models (LM) for three target genes (HSP70, HSC70, Tropomy), from grayling eggs from three different temperatures incubated at two different temperatures (high and low).

a) GLMM HSP70	**Effect**	**Estimate**	**95 % CI**	**P-value**
	Intercept	-0,03	1,44	0,96
	Otta vs. Kvita	0,03	0,88	0,93
	Valåa vs. Kvita	0,07	0,14	0,86
	Temperature	-0,01	0,02	0,93
b) LM HSP70	Intercept	1,19	0,30	<0,001
	Temperature	-0,22	0,28	0,14
	Otta vs. Kvita	-0,08	0,34	0,63
	Valåa vs. Kvita	-0,15	0,34	0,39

a) GLMM HSC70	**Effect**	**Estimate**	**95 % CI**	**P-value**
	Intercept	0,27	1,46	0,7
	Otta vs. Kvita	-0,01	0,90	0,99
	Valåa vs. Kvita	0,08	0,82	0,83
	Temperature	-0,05	0,18	0,55
b) LM HSC70	Intercept	0,92	0,30	<0,001
	Temperature	-0,29	0,28	0,059
	Otta vs. Kvita	0,14	0,34	0,42
	Valåa vs. Kvita	0,12	0,34	0,47

a) GLMM Tropomy	**Effect**	**Estimate**	**95 % CI**	**P-value**
	Intercept	0,73	1,42	0,3
	Otta vs. Kvita	0,02	0,88	0,95
	Valåa vs. Kvita	0,12	0,78	0,75
	Temperature	-0,01	0,16	0,22
b) LM Tropomy	Intercept	1,04	0,34	<0,001
	Temperature	-0,52	0,32	0,006
	Otta vs. Kvita	0,15	0,40	0,47
	Valåa vs. Kvita	0,03	0,40	0,88

Discussion

Recognizing a genes function and how this gene may be affected by external factors can lead to a better understanding of an individual, a population or specie. The main aim of this thesis was to investigate if there were differences in expression of three target genes between populations of European grayling when exposed to two different temperatures during egg and embryo development. The results from the crossing experiment and the monitoring of timing of development in cold rooms did not show a very clear distinction between the populations. Analyzing three target genes, HSC70, HSP70 and Tropomy, that are earlier found to have a large impact on early development and are highly affected by temperature (Huang & Ochiai 2005; Tonsor et al. 2008; Krone et al. 1997), I found no significant differences in the gene expression between the three populations of European grayling incubated at low and high temperatures. What I did find was a significant plastic response in expression of two of the genes, HSC70 and Tropomy. The eggs developing in low temperature (6°C) had a higher expression of the Tropomy gene and HSC70, than the eggs developing in high temperature (10°C).

Timing of Development

There were only small and insignificant differences in the timing of hatching and eye pigment formation between the populations (see fig. 2). From earlier research results (Haugen 2000a, 2000b; Haugen & Vøllestad 2000; Thomassen et al. 2011; Papakostas et al. 2014), I expected differences in development rates between populations, caused by temperature. Previous studies have shown significant temperature effect on development and phenotypes between populations of the Lesja river system. Number of days from fertilization to first observed swim-up have been shown to differ significantly between the populations, which most likely was induced by temperature (Haugen 2000a). Maturation reaction norms have also been shown to differ significantly between populations (Haugen 2000b). Haugen and Vøllestad (2000) presented evidence for differences in growth rate when testing grayling populations in Lesja. They found differences in growth rate between individuals from small and warm streams (SW), and large and cold streams (LC). Larvae from LC populations grew faster than larvae from SW populations under experimental conditions compared to what was observed in the LC population habitats. They also found that the LC population individuals had more efficient yolk-to-body-size transition efficiencies under cold and medium temperature conditions. All of the grayling populations came from the same ancestry originated from fish

spawning in the downstream river Gudbrandsdalslågen, whose temperatures is comparable to the temperature in the SW streams. This indicates that only the individuals from the LC populations had been exposed to temperatures leading to strong selection (Thomassen et al. 2011; Haugen & Vøllestad 2000). Thomassen et al. (2011) presented a study that demonstrated variations between populations of grayling in growth related traits that were significantly influenced by temperature. Grayling from the different populations in Lesja river system responded differently to changes in developmental temperature, with respect to growth in length and yolk-to-body-size transition efficiency (Thomassen et al. 2011). A more recent study of the grayling populations in Lesja also presented phenotypic differences between populations. They discovered that the degree-days to when 50% of the eggs had hatches were higher for the grayling in the cold temperature treatments (Papakostas et al. 2014). In all of these experiments, temperature was an important driving force of adaptation, thus making my results of timing of development the odd one out.

The most obvious reason why differences between populations in timing of hatching and eye pigment formation in my experiment is absent, might be that the common-garden experiment I conducted was not optimally set up to detect such differences. The method of sampling did not produce a variance measure (see Methodical Issues), and thus differences might be hard to detect.

Plastic Responses

The differences in gene expression between populations were absent in my experiment, but differences in gene expression between temperatures were present. I found a plastic response induced by temperature for Tropomy and heat-shock gene HSC70. The expression of Tropomy and HSC70 was significantly higher in all individuals incubated at low temperature (6°C) (see tab. 2). This establishes that the genes (Tropomy and HSC70) are significantly plastic when exposed to different temperature treatments. This is similar to previous studies, which shows that the expression of heat-shock genes, and are highly temperature dependent, (Tonsor et al. 2008; Wu 1995), and studies also show that growth happens more rapidly in cold environments, which is consistent with the finding of a high expression of Tropomy, a gene involved in muscle growth, in low temperature (Kavanagh et al. 2010). Heat-shock genes have been known to increase in expression with elevated temperature (Wu 1995), but my results show a significant increase in expression at lower temperature. A study of insects,

which also are ectothermic, showed that in the flesh flies (*Sarcophagidae*), the up-regulation of heat-shock proteins was essential for cold survival (Denlinger 2002; Flannagan et al. 1998; Rinehart et al. 2006; Yocum et al. 1998). Thus, the heat-shock gene have been proven to be very important for other ectothermic animals, and it is reasonable to say that according to my results, it seems to be important for cold tolerance in grayling as well.

Because of this plastic response in gene expression, I had expected differences between populations, especially among populations where the average temperature is highly different. Two populations were there is big differences in mean water temperature are between Otta and Valåa. Gudbrandsdalslågen (Otta) have a mean summer temperature of 6°C, whilst Kvita is very cold with a mean summer temperature of 2,9°C (Gregersen et al. 2008). These large differences in mean temperature should affect the gene expression between the populations, given that the individuals incubated at low temperature had a significantly higher expression of Tropomy and HSC70. Showing that these two genes are in fact plastic would make them susceptible to local adaptation and genetic differentiation, but when examined they are not. The absent of differences in gene expression between populations might be due to small sample size that could not display differences in gene expression (see Methodical issues).

Genetic Structuring and Gene Expression

The three target genes I studied, Tropomy and heat-shock genes HSC70 and HSP70, showed no differences in expression between the three populations Otta, Kvita and Valåa. One could imply that there are no differences in gene expression or genetic structuring of the populations in Lesja, but earlier studies state otherwise. Several studies show temperature induced genetic differences in development related traits (Thomassen et al. 2011 and references therein). Common-garden experiments have been earlier been conducted, and the differences between the populations are still significant even when they adjust for environmental differences. Haugen and Vøllestad (2000) performed common-garden experiments with three temperatures and measured survival and growth rates during early development and they found significant sire effects, which indicate that the differences have an additive genetic component (Haugen & Vøllestad 2000b). The variation in growth related traits found in a study of Thomassen et al. (2011) was found likely to be genetically based since the results were obtained from a common-environmental experiments. Parallelism in development traits between populations belonging to similar thermal conditions indicates that the differences are consistent with local adaptation to temperature (Thomassen et al. 2011).

Since these experiments resulting in indication of genetic differences between populations, researchers have conducted studies of genetic structure of the grayling populations. These studies have been on neutral components of the DNA (microsatellite F_{ST}), and they have found evidence for genetic differentiation between populations. Researchers has previously found low but significant genetic differentiations among spawning tributaries in Lesja, and thus proving that despite the recent dispersal, genetic structure is developing (Barson et al. 2009; Junge et al. 2011). They found that across all years, a weak but significant indication of genetic structuring based on geographic distance was present (Junge et al. 2011). The F_{ST} estimates were lower between Lesjaskogsvatn and adjacent populations in higher lakes that founded form the Lesjaskogsvatn population, indicating that there has been some gene exchange between the Lesjaskogsvatn demes since colonization of the lake (Barson et al. 2009). Researchers have also shown that early life-history traits can evolve rapidly between grayling populations in Lesja, in spite of conditions that are commonly assumed to limit adaptation (Kavanagh et al. 2010).

The genetic differences exhibited in the recent genetic studies of the grayling populations in Lesja, measure genetic differences on neutral loci and getting a measure of an overall neutral genetic differentiation between populations. When conducting F_{ST} studies one get a measure of the genetic distance between populations due to genetic structuring (Holsinger & Weir 2009; Wright 1930). If F_{ST} is small, it means that the allele frequencies within each population are similar; if it is large, it indicates that the allele frequencies are different (Holsinger & Weir 2009). F_{ST} is a method that examines an overall genetic differentiation between populations, which is the total opposite of what I have done. Searching for differences in gene expression in three specific genes are a much more "needle-in-a-haystack work", focusing in on small parts of the genome. When making decisions in which genes to investigate, I focused on what genes had earlier been proven to differ in expression in early development, but we were also reliable on which primers we had available. This might be the reason why I can't find any differences in gene expression, simply because in these three genes there are no differences in gene expression, yet. One must take into count that these are very young populations and that genetic difference is probably structuring at the moment.

Another reason for why I found no differences between the populations in gene expression could be that the genes are not yet subjected to adaptation, or that they might never be

subjected to adaptation. The level of a genes pleiotropy has been found to have an effect on how a gene responds to selection pressure. In a very recent study, Pakakostas et. al (2014) found that genes with low levels of pleiotropy were very significant during the early phases of evolution. Genes with low pleiotropy had a stronger effect on plastic and evolutionary responses, the level of a genes pleiotropy may play an important role during the early phases of rapid evolution (Papakostas et al. 2014). The three target genes examined in this thesis might be under high level of pleiotropy and thus are not affected by the thermal selection and rapid evolution.

The heat-shock proteins are active in several processes; they function as chaperons, co-chaperons, ligase, are active in processes like apoptosis and protein folding and degradation, and they also might be included in cancer, aging and so on (Heikkila 1993; Gong & Golic 2005; Krone et al. 1997). Whether the heat-shock genes are pleiotropic is debatable, and pleiotropy can be hard to demonstrate (Stearns 2010). If the heat-shock genes cannot be classified as pleiotropic, they are at least very important for numerous cellular processes, and thus very important for the over-all function of the cell (Gong & Golic 2005; Ballinger et al. 1999). Pleiotropy might also affect the Tropomy gene. In chicken embryo fibroblasts that were effected by the Rous sarcoma virus (RSV), the synthesis of tropomyosin had decreased (Hendricks & Weintraub 1981). The decrease in tropomyosin may be a result of pleiotropic effect that results in the transcriptional inactivation not only of the tropomyosin gene, but also the fibronectin and procollagene genes (Hendricks & Weintraub 1981). Paulin et al. (1979). The tropomyosin synthesis seems to be activated by the loss of the transformed phenotype, thus activated by a pleitropic effect (Hendricks & Weintraub 1981).

The hypothesized reason for no gene expression between populations being due to pleiotropy does not correspond to the plastic respond of Tropomy and HSC70. Papakostas et al. (2014) demonstrated that highly pleiotropic genes had limited expression response to temperature treatment. According to this, if the genes were highly pleiotropic, they should not show such differences in gene expression between temperatures. Heat-shock genes HSC70 and Tropomy, had a significantly plastic response to temperature, and the expression of these genes does not seem limited by any causes. This was not the case for heat-shock gene HSP70, so for this gene, pleiotropy might be a reason for the lack of variance in gene expression between populations.

Methodical Issues

The common-garden experiment with observation of development was not optimal. To visualize difference in development traits, I need to have variance in sampling. I did not sample in a way that I got a variance measurement. I sampled ten individuals at each time point, one sampling per time point, per populations, per temperature. Sampling like this will make differences in timing and development hard to detect. The sample size of this experiment was very small (see appendix 5) and this probably had an effect on the statistical analysis. The power and credibility of the GLMM was low due to the low sample size in this thesis. With low sample size, it is hard to draw reasonable conclusions because it has few observations to support it. I ran two plates with qPCR and unfortunately the setup of these two plates were different. On plate one I had an even number of samples over populations and temperatures, while on plate two there was no balance of samples from each populations and temperature (see appendix 6). Some of the individuals used on plate one were used again, but also new individuals were added to the plate two. When having an uneven setup the power of the statistical analysis will get weaker. The reason why I found significant differences between temperatures might be that I did the linear model on only one plate, plate one with the most even setup, and when all populations are considered as one unit, the statistical analysis gets more samples to rely on. When having a larger sample size and an even plate setup for the qPCR, one will have greater power in the statistical analysis and more data to support conclusions drawn from this. I cannot draw any clear conclusions or statement from this thesis; I can only discuss what might be the reason for my results.

Conclusion

To conclude, I did not find significant differences in timing of development, or in gene expression between populations of European grayling from the river system in Lesja. These findings may indicate that the genes are pleiotropic. Highly pleiotropic genes have been found not to have a large effect on evolutionary responses. A second reason that the genes do not show significant expression between the studied populations might be that these are not genes that are suited to find differences, they might not yet be affected, or they might never be effected. To find differences in gene expression in three target genes can be "needle-in-a-haystack-work", because assuming which genes that might have differences in expression during early development can be difficult.

I did find significant differences in gene expression between the two temperatures in two genes; Tropomy and heat-shock gene HSC70, but not for heat-shock gene HSP70. This plastic reaction to temperature provides evidence that the gene expression of Tropomy and HSC70 are influenced highly by temperature. According to this result I would expect difference between the populations in gene expression, especially between Otta and Valåa, since the mean temperature differences between the former and the latter populations are highly different. Finding differences in gene expression between two temperatures indicates that the heat-shock protein HSC70 are important for cold tolerance. Tropomy, a gene involved in muscular growth, was also up regulated, which is consistent with other studies on growth in cold habitats versus warmer habitat. No proper conclusions can be drawn from these results, since the sample number was low and sampling was conducted in a way that does not provide a variance measurement.

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Appendix

Appendix 1: Date of sampling gamets, transportation from population location to lab, when crossing experiment was conducted, and how many individuals used in the crossing experiment for each location/population. See figure 1 for population locations.

Location	Field Sampling	Arrived UiO	Crossing experiment	Females/Males
Otta	05.06 2013	05.06 2013	05.06 2013	4f/6m
Steinbekken	07.06. 2013	07.06 2013	07.06 2013	2f/2m
Kvita	13.06 2013	14.06 2013	14.06 2013	4f/5m
Valåa	14.06 2013	14.06 2013	14.06 2013	5f/fm
Hårrtjønn	16.06 2013	16.06 2013	17.06 2013	5f/5m

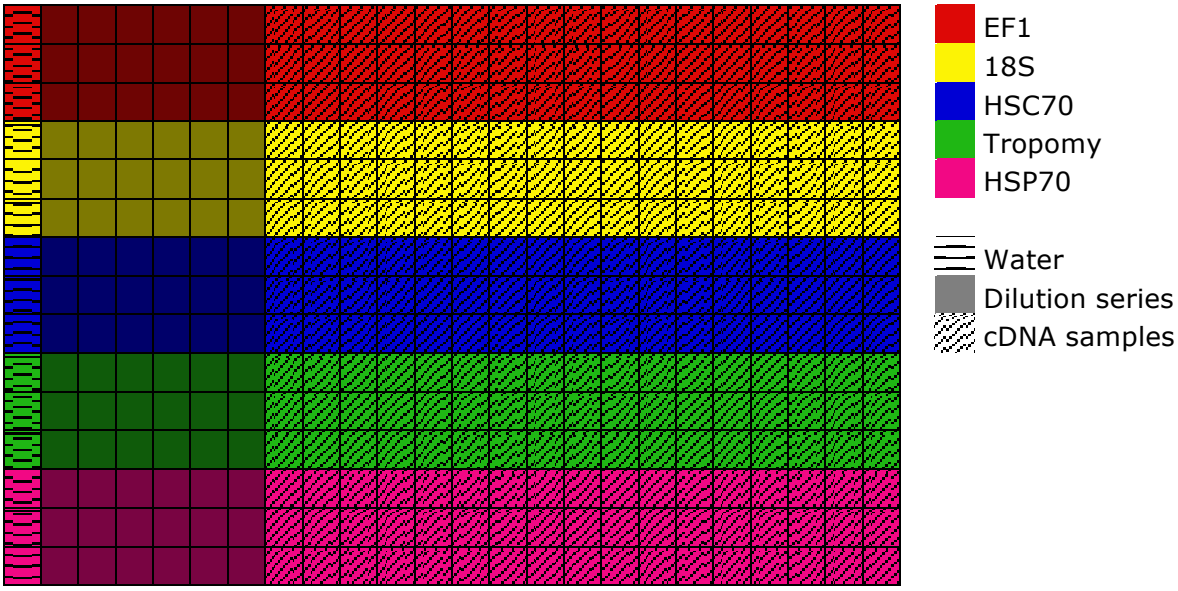
Appendix 2: Sampling date and time: Ten eggs were sampled at each sampling point (I: eye pigm. II: St. dd. III: St. dd. IV: Hatching), for all of the populations. The dates are when sampling was done i.e. when eye pigment became visual, after a set number of degree-days, and the day the eggs hatched.

Target temp	Sample	Otta	Steinbekken	Kvita	Valåa	Hårrtjønn
10°C	I	17.06 11:30	18.06 13:30	25.06 14:00	25.06 14:00	27.06 13:00
10°C	II	19.06 12:30	21.06 12:30	28.06 16:00	28.06 13:00	30.06 18:00
10°C	III	21.06 12:00	23.06 15:00	30.06 17:00	30.06 17:00	02.07 13:00
10°C	IV	23.06 16:00	23.06 15:30	02.07 13:30	02.07 13:30	03.07 15:00
8°C	I	20.06 12:00	23.06 16:00	28.06 15:30	28.06 15:00	01.07 13:00
8°C	II	22.06 15:00	24.06 10:30	01.07 13:00	01.07 13:00	03.07 14:00
8°C	III	25.06 14:30	27.06 13:00	04.07 11:00	04.07 11:00	06.07 13:00
8°C	IV	04.07 10:30	04.07 10:30	09.07 14:00	09.07 13:00	10.07 15:00
6°C	I	23.06 15:30	25.06 14:30	01.07 13:30	01.07 13:30	03.07 14:00
6°C	II	28.06 15:30	30.06 17:00	07.07 14:30	07.07 14:30	09.07 14:30
6°C	III	02.07 13:00	04.07 11:00	11.07 15:00	11.07 15:00	13.07 16:00
6°C	IV	04.07 11:00	04.07 11:00	11.07 15:30	11.07 15:00	14.07 19:30

Appendix 3: Degree-days used for each population and temperature to get to each time point. I: Eye pigment formation, II: Fixed degree-day near eye pigment formation, III: Fixed degree-day near hatching, and IV: hatching.

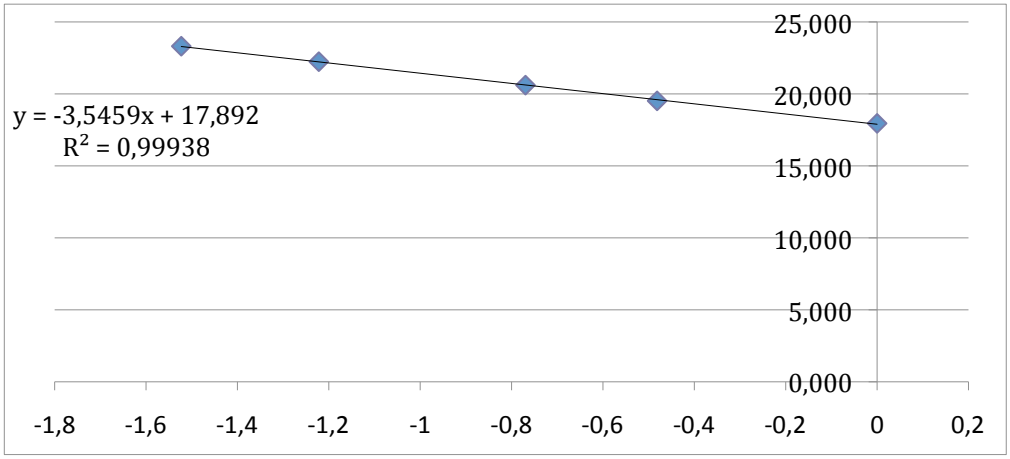
Temperature	Timepoint	Otta	Steinbekken	Kvita	Valåa	Hårrtjønn
10	I	134	123,7	123,7	123,7	134
10	II	144,2	144,2	144,2	144,2	164,8
10	III	175,1	175,1	175,1	175,1	185,4
10	IV	195,7	175,1	197,7	197,7	195,7
6	I	127,2	127,2	120,5	120,5	127,2
6	II	160,7	160,7	160,7	160,7	167,5
6	III	187,8	187,2	181,1	187,8	194,6
6	IV	201,3	187,2	187,8	187,8	201,3

Appendix 4: Example of plate setup (plate 1). Each vertical row of squares represents a separate sample of cDNA

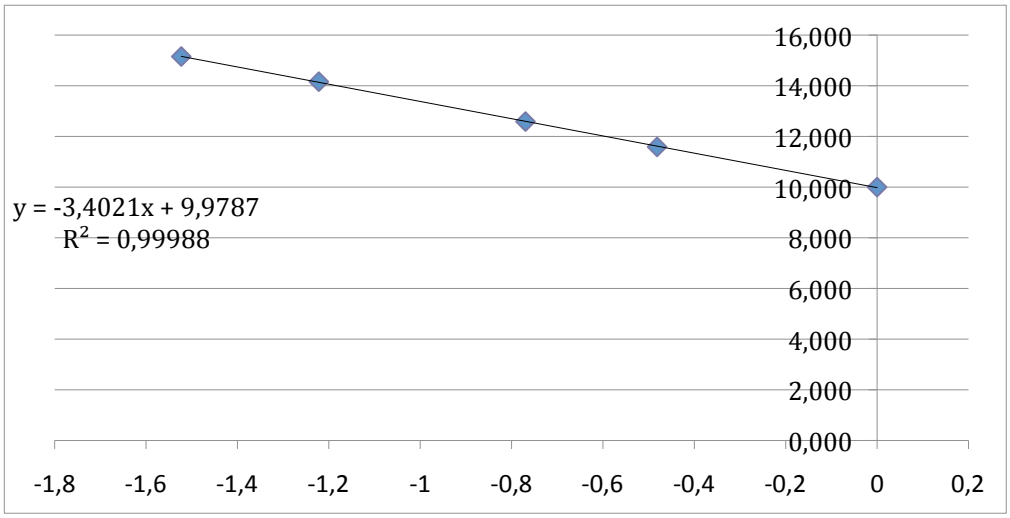


Appendix 5: Standard curves from qPCR dilution series for two reference genes and the three target genes used in calculation of the relative mRNA values.

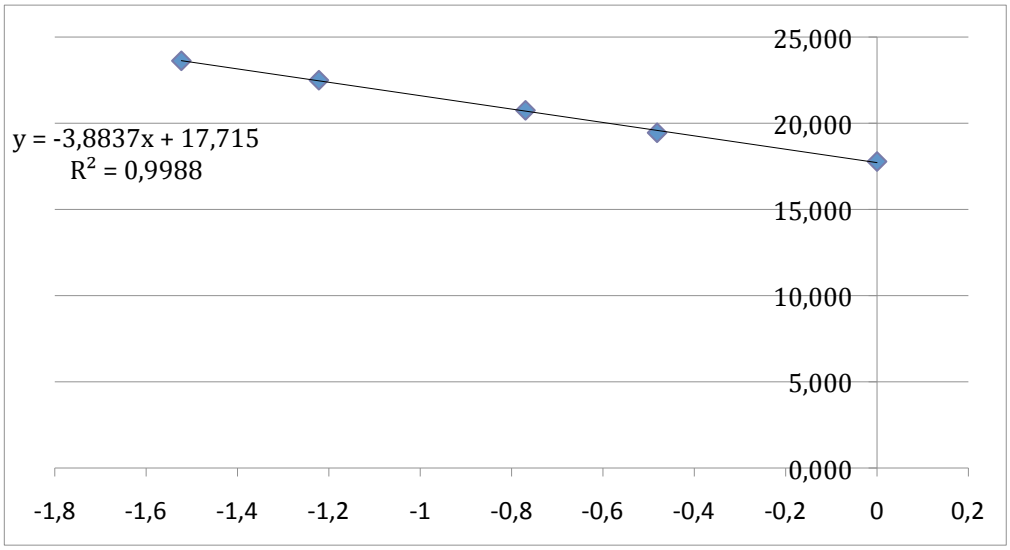
Standard curve for EF1



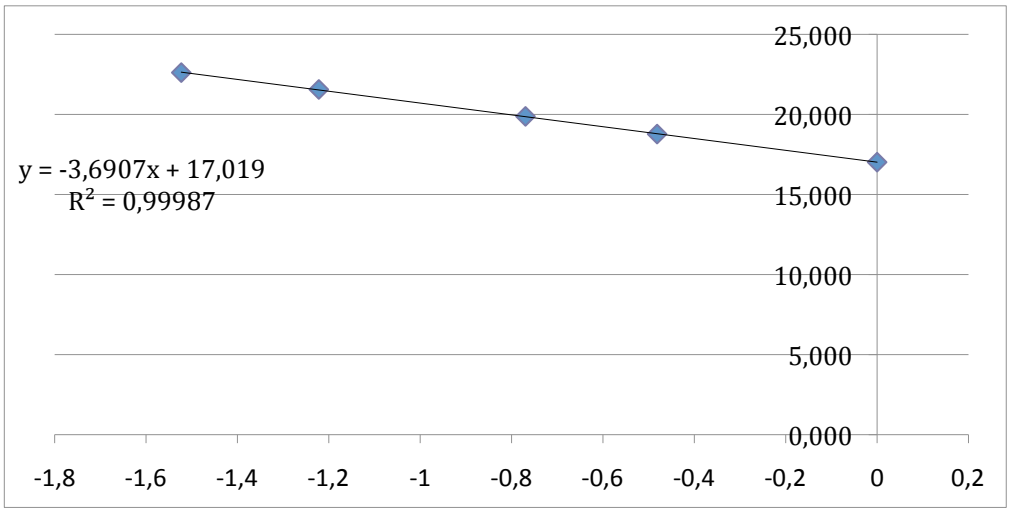
Standard curve for 18s



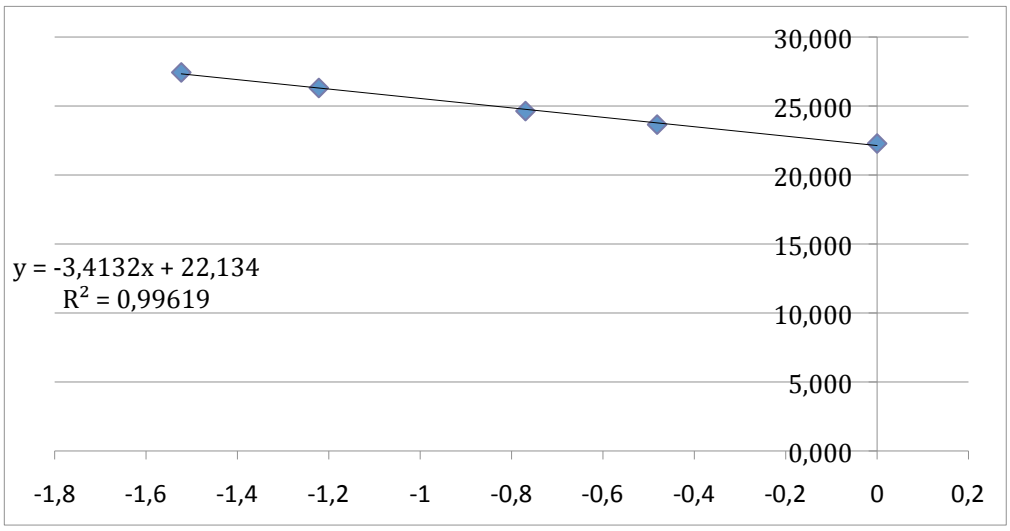
Standard curve for HSC70



Standard curve for Tropomy



Standard curve for HSP70



Appendix 6: The data set used for statistical analysis. From left: Individual sample, on which plate it was analyzed, the population, the temperature, and the relative mRNA value for each individual sample for the three target genes HSC70, HSP70 and Tropomy

Individual	Plate	Population	Temperature	HSC70	HSP70	Tropomy
KV4 6	2	Kvita	6	0,785528033	0,907492301	1,084016835
KV10 6	2	Kvita	6	0,802630744	0,953922466	0,919940109
KV 7 6	2	Kvita	6	1,125433808	0,744263466	1,12504174
KV 8 6	2	Kvita	6	0,963268689	0,570915034	1,366005618
KV 9 6	2	Kvita	6	1,366863059	0,665404462	1,321321206
KV 8 6	1	Kvita	6	0,73048621	0,831263143	0,876521285
KV9 6	1	Kvita	6	1,039061625	1,929505461	1,172929241
KV 5 10	2	Kvita	10	0,966736269	1,231648701	1,005649163
KV 6 10	2	Kvita	10	0,8052448	0,52464838	0,761087692
KV6 10	1	Kvita	10	0,538257916	0,725839593	0,457021705
KV8 10	1	Kvita	10	0,628654778	0,935009407	0,511778464
KV9 10	1	Kvita	10	0,786349726	0,896927792	0,624302643
VA4 6	2	Valaa	6	0,750644794	0,988178343	0,866478507
VA5 6	2	Valaa	6	0,64602501	0,917853925	0,703878042
VA10 6	2	Valaa	6	0,951779911	1,300402437	1,100970334
VA7 6	2	Valaa	6	1,836236278	0,514825068	3,1727065
VA6 6	1	Valaa	6	1,710884498	1,153262305	1,933960732
VA8 6	1	Valaa	6	0,902372389	1,007855864	0,762523871
VA9 6	1	Valaa	6	0,497833879	0,647618985	0,611734485
VA3 10	2	Valaa	10	0,973578827	1,178166676	1,116620463
VA4 10	2	Valaa	10	1,003195609	1,179900972	0,996508056
VA5 10	2	Valaa	10	0,982284386	1,085062557	0,816663922
VA10 10	2	Valaa	10	0,640447747	0,857548538	0,720371027
VA6 10	1	Valaa	10	0,566175083	0,723186811	0,525471648
VA8 10	1	Valaa	10	0,801645642	0,983417439	0,533119794
VA9 10	1	Valaa	10	0,939261488	1,102882253	0,496321408
OT4 6	2	Otta	6	1,022344269	1,020213906	1,124388756
OT5 6	2	Otta	6	0,610658998	0,828851816	0,648718076
OT10 6	2	Otta	6	0,663305092	0,841766083	0,791485833
OT6 6	1	Otta	6	1,40450634	0,980303168	1,566071102
OT 8 6	1	Otta	6	1,050828645	1,110943805	1,10786284
OT9 6	1	Otta	6	0,853325175	1,201854789	0,844352358
OT7 10	2	Otta	10	0,983275118	0,727028675	1,520090745
OT8 10	1	Otta	10	0,752758931	0,992159093	0,723461834
OT9 10	1	Otta	10	0,733614137	0,898999923	0,604140599
OT10 10	1	Otta	10	0,739471548	0,830979149	0,723257732